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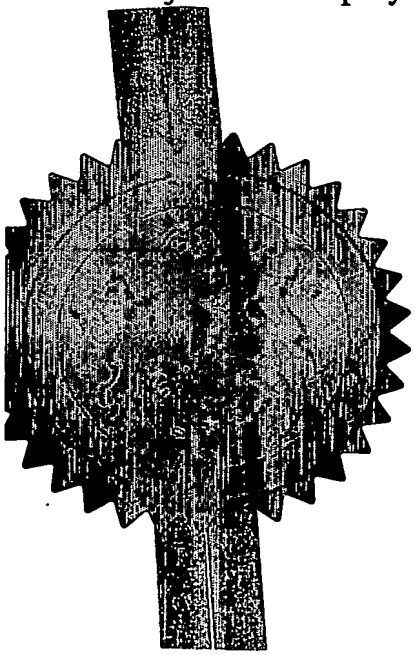

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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Imperial College Innovations Limited
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E852904-4 D02866
P01/7700 0.00-0326780.4

Patents ADP number *(if you know it)* 74094360004

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — BIOLOGICAL MATERIALS AND USES THEREOF

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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BIOLOGICAL MATERIALS AND USES THEREOF

The presently claimed invention relates to methods of identifying and/or making compounds for use in the reduction and/or prevention of fibrosis. The invention also relates to compounds for reducing and/or preventing fibrosis and to the use of such compounds.

When biological tissue is injured, both the injury and the associated inflammatory response can cause the death of cells. When cell death occurs, new tissue is synthesised to replace the dead or dying cells. The synthesis of new tissue falls under two categories, the regeneration of specialised cells and an increase in connective tissue. In some pathological conditions, the connective tissue increase dominates the healing process, leading to the formation of fibrotic tissue. In fibrosis, the new tissue has repaired any structural defect in the tissue, but has impaired its own function by replacing the specialised cells by connective tissue and connective tissue-producing cells.

Whether fibrosis occurs, or indeed the extent of the fibrosis, is influenced by a variety of factors, including the nature, severity and location of the injury to be healed. Fibrosis is most commonly known as scars on the surface of the skin, where it is relatively un-troublesome, except in scarring over large areas. However, fibrosis can also occur in the tissues of internal organs e.g. liver, lung and kidney. In most cases, it is fibrosis in these areas that is most serious because the specialised activity of that organ is impaired. In the most extreme cases organ failure or death can occur because of that impairment.

An example of the importance of fibrosis in the disease-state is demonstrated by the occurrence of fibrosis of the kidney (diabetic nephropathy) in diabetes mellitus, a disease now reaching epidemic proportions worldwide.

The incidence of diabetes mellitus has undergone a global increase in recent years. In particular this is due to a dramatic increase in type 2 diabetes (late-onset diabetes) (Silink M (2002), *Horm. Res.* **57** (Suppl 1) pp. 1-5). Diabetes mellitus is closely linked to a number of secondary complications, especially microvascular related complications. These complications, including the fibrotic condition nephropathy, usually develop a number of years after the onset of diabetes.

Diabetic nephropathy is characterised by excessive deposition of extracellular matrix proteins in the mesangium and basement membrane of the glomerulus and in the renal tubulointerstitium.

Genetic background is thought to be important in determining susceptibility to diabetic nephropathy (DN) (Quinn M *et al.*, (1996) *Diabetologica* **39** pp 940-945), but the crucial initiating factor is believed to be exposure of tissues to chronic hyperglycaemia (UKPDS Group, (1998) *Lancet* **352** pp. 837-853). The prevalence of nephropathy varies according to geographical location, type of diabetes, and the length of time since diagnosis. Notwithstanding influencing factors, the prevalence of diabetic nephropathy is predicted to increase in the decades ahead (Bagust A *et al.* (2002) *Diabetes Med* **19** (Suppl 4): pp1-5). Diabetic nephropathy is a major cause of end-stage renal disease, and new therapeutic approaches are required to limit its development.

The pathology of diabetic nephropathy is similar in types 1 and 2 diabetes. Both types of diabetes are associated with similar ultrastructural changes occurring in kidney glomeruli (Osterby R, (1992) *Diabetologica* 35 pp 803-812). The glomerular basement membrane increases in thickness, and the extracellular matrix of the mesangium expands.

It is expansion of the mesangium that is thought to be the main cause of reduced renal function in diabetic nephropathy (Steffes M *et al.* (1989) *Diabetes* 38 pp1077-1081). As the mesangial matrix expands, it impinges on glomerular capillaries, reducing the surface available for filtration and narrowing or occluding the lumen. Tubulointerstitial fibrosis also occurs in diabetic nephropathy, in addition to glomerulosclerosis. The progressive loss of renal function correlates with the occurrence of advancing interstitial fibrosis in other renal disorders (Risdon R *et al.* (1968) *Lancet* 2 7564 pp363-366).

Fibrotic disease is commonly associated with an imbalance in growth factors and hormones, which in turn influence the production of protein expression. The abnormal protein expression in turn leads to the formation of fibrosis. For example, fibrosis is commonly influenced by an increase in transforming growth factor-β present in the fibrotic tissue.

Fibrosis is one of the largest groups of disorders for which there is no effective therapy, in part because the mechanism underlying these disorders is influenced by a variety of factors and exact cellular mechanisms have not been elucidated. Therefore, there is a lack of understanding of which, or the nature of molecular targets which may provide targets around which anti-fibrotic therapies may be based.

In the case of diabetic nephropathy, studies have shown that glucose can induce matrix synthesis, at least in part, by the actions of transforming growth factor-β (TGF-β) (Ziyadeh F *et al.* (2000) *Proc Natl Acad Sci USA* **97** pp. 8015-8020).

However, TGF-β has a number of physiological roles including involvement in immunity and epithelial proliferation (McCartney-Francis N *et al.* (1998) *Int. Rev. Immunol.* 16 pp. 553-580). These varying physiological effects mean that TGF-β is unlikely to be a clinically advantageous target. Blocking the actions of TGF-β may have multiple effects on the organism, causing unwanted and potentially serious side effects.

Transforming growth factor-β causes fibrosis by the direct induction of collagen and matrix synthesis. Additionally, TGF-β is also able to induce the expression of other molecules that take part in and/or influence the pathways causing fibrosis. One such protein is connective tissue growth factor (CTGF), which induces proliferation, collagen synthesis and chemotaxis in mesenchymal cells (Moussad E *et al.* (2000) *Molec Genet Metab.* **71** pp.276-292). CTGF (CCN2) is a 38 kDa secreted protein with multiple domains, encoded by an immediate-early gene and is a member of the CCN protein family (Bork *et al.* (1993) *Febs Lett.* **327** pp 125-130; Perbal *et al.* (2001) *Mol. Pathol.* **54** pp 57-79). However, the molecular mechanism(s) by which it functions have not been fully elucidated. The presence of multiple domains in CTGF suggests that it interacts with a plurality of other factors. CTGF has been shown to directly bind BMP4 and TGF-β through its von Willebrand type C domain, leading to inhibition of BMP and enhancement of TGF-β signalling (Abreu *et al.* (2002) *Nat. Cell. Biol.* 4 pp. 599-604).

CTGF has also been shown to bind to integrins (Babic *et al.* (1999) *Mol. Cell Biol.* **19** pp.3811-3815) and it is possible that this interaction is important in mediating some of the cellular phenomena that CTGF induces.

CTGF is over-expressed in a variety of fibrotic disorders, including diabetic nephropathy (Wahab N *et al.* (2001) *Biochem J.* **359** pp.77-87). In fact, increasing levels of CTGF expression have been shown to correlate with increasing severity and speed of progression of diabetic nephropathy (Ito Y *et al.* (1998) *Kidney Int.* **53** pp.853-886).

Hence, CTGF may be a potentially useful molecular indicator of the fibrotic response. CTGF has not yet been shown to directly induce renal fibrosis in vivo, but, when injected subcutaneously along with TGF-β, induces sustained dermal fibrosis in rats (Mori T *et al.* (1999) *J. Cell. Physiol.* **181** pp 153-159).

In the process of developing this invention, the inventors have demonstrated that CTGF interacts with a cellular receptor, the TrkA receptor, in order to induce intracellular signalling cascades related to the formation of fibrosis.

There are three Trk receptor tyrosine kinase genes (TrkA, TrkB and TrkC). On binding its ligand, the Trk receptor dimerizes and autophosphorylates, leading to the activation of several small G proteins, including Ras, Rap-1, and the Cdc 42-Rac-Rho family, as well as of pathways regulated by MAP kinase, PI3-kinase, and phospholipase C-γ (PLC γ) (Segal, 2003). Activated Trk receptors also interact, directly or indirectly, with a variety of cytoplasmic adaptor proteins to produce a number of biological responses including, cell proliferation and survival; axonal and dendritic growth, and

remodelling; assembly and remodelling of cytoskeleton; membrane trafficking and fusion; and synapse formation, function, and plasticity (Huang E and Reichardt L, (2003) *Annu. Rev. Biochem.* 72 pp. 609-642).

The work by the inventors described in the examples, has shown that Trk tyrosine kinase activity is required for the CTGF-dependent induction of intracellular signalling molecules implicated in fibrosis. This work has led to the establishment of a method of identifying and making compounds that can interact with the CTGF receptor and/or an agonist of the CTGF receptor in order to reduce or prevent fibrosis.

Therefore, in a first aspect of the invention is provided a method for identifying and/or making compounds for use in reducing and/or preventing fibrosis, comprising the steps:

(a) providing a CTGF receptor

(b) providing a test sample

(c) providing a CTGF receptor agonist

(d) exposing the CTGF receptor to the test sample

(e) subsequently or simultaneously exposing the CTGF receptor to the CTGF receptor agonist

(f) detecting and/or measuring the amount of CTGF receptor activation

(g) comparing the amount of CTGF receptor activation in the presence of a test sample to the amount of CTGF receptor activation detected and/or measured in the absence of a test sample;

(h) determining if a compound reduces and/or prevents fibrosis on the basis that it causes no increase or a decrease in CTGF receptor activation.

By "CTGF receptor agonist" we mean a compound acting at the CTGF receptor to produce an effect that is substantially the same as that of the effect produced by CTGF interacting with the receptor. We also include derivatives, analogues and fragments of CTGF receptor agonists that are capable of producing substantially the same effect as CTGF interacting with the CTGF receptor. CTGF receptor agonists, other than CTGF itself, can be readily identified by measurement and/or detection of CTGF receptor autophosphorylation, receptor induced protein phosphorylation and TIEG expression using the methods presented in Example 1.

By "derivative" we mean a CTGF receptor agonist compound, additionally having at least one chemical modification of one or more of its amino acid side groups, a-carbon atoms, terminal amino group, or terminal carboxylic acid group. A chemical modification includes adding chemical moieties, creating new bonds, and removing chemical moieties. Modifications at amino acid side groups include acylation of lysine e-amino groups, N-alkylation of arginine, histidine, or lysine, alkylation of glutamic or aspartic carboxylic acid groups, and deamidation of glutamine or asparagine. Modifications of the terminal amino include the des-amino, N-lower alkyl, N-di-lower alkyl, and N-acyl modifications. Modifications of the terminal carboxy group include the amide, lower alkyl amide, dialkyl amide, and lower alkyl ester modifications. A lower alkyl is a C1 -C4 alkyl. Furthermore, one or more side groups, or terminal groups, may be protected

by protective groups known to the ordinarily skilled protein chemist. The I-carbon of an amino acid may be mono- or di-methylated.

By "analogue" we mean a CTGF receptor agonist having a modification including one or more amino acid substitutions, deletions, inversions, or additions and capable of producing an effect that is substantially the same as that of the effect produced by CTGF interacting with the receptor.

By "fragment" we mean a portion of a CTGF receptor agonist capable of producing an effect that is substantially the same as that of the effect produced by CTGF interacting with the receptor.

Optionally the method further comprises the step of isolating the compound which is capable of reducing and/or preventing fibrosis. The isolated compound may then optionally be formulated into a composition further comprising a pharmaceutically acceptable carrier, excipient and/or diluent.

Preferably, CTGF receptor activation is detected and/or measured by detecting and/or measuring at least one of the following activities: CTGF receptor autophosphorylation, CTGF receptor-induced protein phosphorylation or CTGF induced expression of TIEG. Typical methods of measuring these activities are provided in Examples 1 and 2.

Preferably the CTGF receptor agonist is CTGF.

Preferably the CTGF receptor is the TrkA receptor.

Conveniently the compound affects directly the interaction between the CTGF receptor and an agonist thereof. In other words, the compound

interacts directly with the CTGF receptor or agonist thereof in order to reduce the activation of the CTGF receptor.

Alternatively, the compound affects indirectly the interaction between the CTGF receptor and an agonist thereof. In other words, the compound interacts with the CTGF receptor or agonist thereof indirectly via at least one further compound in order to reduce the activation of the CTGF receptor.

Conveniently, the compound identified and/or made by the method described above is an antagonist of a tyrosine kinase.

By "antagonist" we mean a compound acting at the CTGF receptor to inhibit and/or prevent the effect produced by CTGF or a CTGF receptor agonist interacting with the receptor. We also include derivatives, analogues and fragments of CTGF receptor antagonists that are capable of preventing and/or inhibiting the effect of CTGF and/or a CTGF receptor agonist interacting with the CTGF receptor. CTGF receptor antagonists, can be readily identified by measurement and/or detection of CTGF receptor autophosphorylation, receptor induced protein phosphorylation and TIEG expression using the methods presented in Example 1.

In a second aspect of the invention there is provided a compound for use in the reduction and/or prevention of fibrosis characterised in that it inhibits and/or prevents CTGF receptor activation; and more preferably inhibits and/or prevents at least one of the following activities: CTGF receptor autophosphorylation; CTGF receptor-induced protein phosphorylation; and/or induction of TIEG.

Preferably the compound is identified and/or made by the method of the first aspect of the invention.

Conveniently, the compound is at least one selected from polypeptides, antibody molecules and antisense nucleotides. Preferably the compound is an antibody molecule.

The term "antibody molecule" shall be taken to refer to any one of an antibody, an antibody fragment, or antibody derivative. It is intended to embrace wildtype antibodies, synthetic antibodies, recombinant antibodies or antibody hybrids, such as, but not limited to, a single-chain modified antibody molecule produced by phage-display of immunoglobulin light and/or heavy chain variable and/or constant regions, or other immunointeractive molecule capable of binding to an antigen in an immunoassay format that is known to those skilled in the art.

The term "antibody derivative" refers to any modified antibody molecule that is capable of binding to an antigen in an immunoassay format that is known to those skilled in the art, such as a fragment of an antibody (e.g. Fab or Fv fragment), or a modified antibody molecule that is modified by the addition of one or more amino acids or other molecules to facilitate coupling the antibodies to another peptide or polypeptide, to a large carrier protein or to a solid support (e.g. the amino acids tyrosine, lysine, glutamic acid, aspartic acid, cysteine and derivatives thereof, NH_2-acetyl groups or COOH-terminal amido groups, amongst others).

By "antisense oligonucleotides" we mean single-stranded nucleic acids, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA-RNA, a DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed

"antisense" because they are complementary to the sense or coding strand of the gene. Recently, formation of a triple helix has proven possible where the oligonucleotide is bound to a DNA duplex. It was found that oligonucleotides could recognise sequences in the major groove of the DNA double helix. A triple helix was formed thereby. This suggests that it is possible to synthesise sequence-specific molecules which specifically bind double-stranded DNA *via* recognition of major groove hydrogen binding sites.

By binding to the target nucleic acid, the above oligonucleotides can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking the transcription, processing, poly(A)addition, replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradations.

Antisense oligonucleotides are prepared in the laboratory and then introduced into cells, for example by microinjection or uptake from the cell culture medium into the cells, or they are expressed in cells after transfection with plasmids or retroviruses or other vectors carrying an antisense gene.

Alternatively, the compound is a tyrosine kinase receptor inhibitor. Preferably the tyrosine kinase inhibitor is selected from the group consisting of BSF-466895, AP-23451, AP-23464, AP-23485, AZD-0530, AP-22408, RG-13022, RG-13291, RG-14620, RP 53801, CEP-075, CEP-2563 dihydrochloride, CHIR-200131, CHIR-258, c-jun kinase, KST-638, KF-250706, MNAC-13, anti-EphA2 Mabs, MLN-608, AG-957, lavendustin A analogues, NSC-330507, NSC-680410, phenylalanine derivatives, SH2 inhibitors, AG-1295, EGF-genistein, erbstatin, genistein, neuT Mab, PP1, TT-232, CGP-52411, CGP-53716, CGP-57148, imatinib, NVP-AAK980-

NX, NV-50, phenoxodiol, FAK inhibitors, IGF-1 , Met receptor inhibitors, TIE-2 inhibitors, CP-564959, PN-355, CP-127374, FCE-26806, FGFR-3 inhibitors, Met RTK antagonists, PD-171026, PD-173956, PD-180970, Src non-RTK antagonists, kahalalide F, CCX2, celastrol, TAK-165 ,TG-100-13, TG-100-96, desmal, U3-1566 and SKI-606.

Preferably the compound is a CTGF receptor antagonist.

In a third aspect of the invention there is provided a compound of the second aspect of the invention for use in the treatment and/or prevention and/or diagnosis of a fibrotic disease.

Preferably the compound of the second aspect of the invention is used in the manufacture of a medicament for the treatment and/or prevention and/or diagnosis of a fibrotic disease.

Conveniently the fibrotic disease is one selected from diabetic nephropathy, non-diabetic kidney fibrosis, lung fibrosis, liver fibrosis (cirrhosis), skeletal muscle fibrosis, cardiac muscle fibrosis, atherosclerosis, systemic sclerosis, scleroderma, retinal fibrosis, radiation fibrosis, keloid scar formation and cancer-associated fibrosis

Preferably the disease is diabetic nephropathy.

In a fourth aspect of the invention there is provided a method of treating and/or preventing fibrotic disease comprising administering a therapeutically or prophylactically effective dose, or plurality of doses, of a compound identified and/or made according to the method of the first aspect of the invention.

Conveniently the fibrotic disease is one selected from diabetic nephropathy, non-diabetic kidney fibrosis, lung fibrosis, liver fibrosis (cirrhosis), skeletal muscle fibrosis, cardiac muscle fibrosis, atherosclerosis, systemic sclerosis, scleroderma, retinal fibrosis, radiation fibrosis, keloid scar formation and cancer-associated fibrosis

Preferably the disease is diabetic nephropathy.

Examples embodying certain preferred aspects of the invention will now be described with reference to the following figures in which:-

Figure 1 - CTGF activates intracellular signalling pathways

Serum-starved human mesangial cells (HMC) incubated in the presence of CTGF/V5 fusion protein for the periods of time indicated. Equal amounts of cellular lysate protein were subjected to SDS-PAGE and analysed by Western blotting using phospho-specific antibodies against the constituent proteins of (A) the MAPK pathway, (B) JNK, and (C) PKB and CamKII. β-actin is shown as a marker for equal protein loading. D) Cells were grown on coverslips and serum-starved for 48 hours prior to incubation in medium in the absence (a) and (c), or presence of 40 ng/ml CTGF-fusion protein (b) and (d) for 30 min. Cells were fixed, permeabilized, probed with anti-phospho PKC δ (a) and (b) and PKC α (c) and (d) primary antibodies, and then with fluorescein-conjugated secondary antibody. Results are representative of three separate experiments.

Figure 2 - CTGF induces tyrosine phosphorylation of different proteins

Serum-starved HMC were incubated in the presence of 40 ng/ml CTGF/V5 fusion protein for the periods of time indicated. Equal amounts of cellular

lysate protein were subjected to SDS-PAGE and analysed by Western blotting using anti-phosphotyrosine antibody. Results are representative of three separate experiments.

Figure 3 - CTGF interacts with HMC surface proteins

CTGF/V5 fusion protein was allowed to bind to the cell surface, and then chemically cross-linked to its ligands with BS^3, after which a membrane-enriched fraction was prepared from the cells. Cross-linked CTGF complexes were immunoprecipitated using rabbit anti-CTGF antibody, resolved by SDS-PAGE, and analysed by Western blotting using chicken anti-CTGF antibody (lane 2). The cross-linking step was omitted for some cultures (lane 1). Results are representative of three separate experiments.

Figure 4 - CTGF interacts with TrkA and p75NTR in HMC

(A) Serum-starved HMC were incubated in the absence (lane 1) or presence (lane 2) of CTGF/V5 fusion protein (40 ng/ml) for 15 min, after which cell lysates were prepared in RIPA buffer. Equal amounts of lysate protein were immunoprecipitated using anti-phosphotyrosine beads. Bound proteins were resolved by SDS-PAGE and analysed by Western blotting using an antibody against TrkA.

(B) HMC were incubated in the absence (lane 1) or presence (lane 2) of His tagged-CTGF/V5 fusion protein (200 ng/ml) for 2 h at 4°C to allow binding to cell surface receptors, after which the protein was chemically cross-linked with DTSSP. A membrane-enriched fraction was prepared and solubilised. Equal amounts of solubilised protein were incubated with metal affinity beads. Bound proteins were subjected to SDS-PAGE

under reducing conditions, and Western blotting using an antibody against TrkA

(C) HMC were incubated with 200 ng/ml rCTGF (FibroGen Inc.) for 2 hours at 4°C. Bound CTGF was cross-linked as above, and a membrane-enriched fraction prepared and solubilised. Cross-linked CTGF-complexes in the solubilised fraction were captured on anti-C-terminus-CTGF antibody affinity beads (lane 1), or the fraction was incubated with control IgG affinity beads (lane 2). Bound proteins were analysed by Western blotting using anti-TrkA antibody.

(D) The sample shown in lane 1 of Figure 4C, was boiled for a longer time and then Western blotted using anti-TrkA antibody.

(E) Blot (D) was stripped and re-probed using anti-p75NTR antibody.

Results are representative of 4 separate experiments.

Figure 5 - HMC expresses Trk receptors

Total RNA was extracted from HMC and used for RT-PCR. After amplification, 10 µl of each PCR reaction product was electrophoresed through a 1.2% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Results are representative of three separate experiments.

Figure 6 - CTGF activates TrkA in HMC

Serum-starved HMC were incubated in the absence (lane 1) or the presence (lane 2) of CTGF/V5 (40 ng/ml) for 15 min. Equal amounts of cellular lysate protein were subjected to SDS-PAGE and analysed by Western

blotting. Blot A was probed with anti-TrkA antibody. Blot B was probed with anti-phospho-TrkA (Tyr490) antibody, while blot C was probed with anti-phospho-TrkA (Tyr674/675). Results are representative of three separate experiments.

Figure 7 - Stimulation of TIEG levels by CTGF in HMC

Serum-starved HMC were exposed to rCTGF/V5 fusion protein for different periods of time, after which cell lysates were prepared and the TIEG and b-actin levels analysed by Western blotting. A representative blot of the three independent experiments (three replicate cultures per condition per experiment) that were performed is shown.

Figure 8 - TIEG mediates CTGF-dependent down-regulation of Smad 7 expression level.

Serum-starved HMC were exposed to the conditions indicated in the figure. After 24 h, cell lysates were prepared, and the TIEG, Smad 7, and b-actin levels analysed by Western blotting. A representative blot of the three independent experiments (three replicate cultures per condition per experiment) that were performed is shown.

EXAMPLES

Example 1 – Identification of CTGF – Trk A interaction

Materials and Methods

Cell cultures, antibodies and reagents

Primary normal adult human mesangial cells (HMC) (CC-2259, lot 3F1510) (Biowhittaker, Wokingham, Berkshire, U.K.) were maintained in culture as described previously (Wahab N *et al.* (1996) *Biochem J.* **316** pp.985-992).

Confluent post-exponential-phase cultures of HMCs (passage 6-8) were maintained in culture medium containing 10% (v/v) foetal calf serum and 4 mM (normoglycaemic), 11, 15 or 30 mM (hyperglycaemic) d-glucose for periods of up to 4 weeks. At the end of each week cultures were washed extensively with PBS and were used either for RNA extraction or for culture for 24 h in glucose supplemented medium in the absence of serum.

Phospho-Akt antibody (P-Ser 472/473/474) (Pharmingen, San Diego, CA, USA), Phospho-Akt (P-Thr 308) (Sigma, Gillingham, Dorset, UK) and ERK5 antibodies (Sigma, Gillingham, Dorset, UK) were used.

Phospho-ERK1/2 pathway sampler, phospho-JNK pathway sampler, phospho P38 MAPK pathway sampler, phospho-PKC δ, phospho-PKC α, phospho-TrkA (Tyr674/675), phospho-TrkA (Tyr490) antibodies were from New England BioLabs (Hitchen, Herts., UK). Phospho-CaMKII (P-Thr286) antibody was from Promega (Southampton, Hants., UK) and anti-phospho-tyrosine antibody was from Santa Cruz (Autogen Bioclear, Calne, Wilts., UK). Anti-TrkA antibody was obtained from Upstate Biotechnology

(Milton Keynes, UK). Anti-TIEG-1 antibody was a gift from Dr. Steven Johnson (Mayo Foundation, Minnesota, USA). K-252a was purchased from Calbiochem (Nottingham, UK). Recombinant CTGF (CTGF/V5 fusion protein) was expressed in transformed HMC and purified from the medium using Talon metal affinity resin, (Wahab N *et al.* (2001) *Biochem J.* **359** pp.77-87). Alternatively, r-CTGF (non-fusion protein) was expressed in the baculovirus system and was a gift from FibroGen Inc. (South San Francisco, CA, USA). Rabbit anti-CTGF (pAb2) and chicken anti-CTGF (pIgY3) were also supplied by FibroGen Inc.

Cross linking and membrane preparation

Cell layers were washed twice with cold binding buffer (PBS and 0.5% glucose) and incubated with CTGF in binding buffer for 2 h at 4°C. After incubation, the cell layers were washed five times with cold binding buffer and incubated with 1mM 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) or disuccinimidyl suberate (DSS) (Pierce Biotechnology, Tattenhall, Cheshire, UK) in PBS for 30 min at room temperature. The reaction was halted for 15 min at room temperature by the addition of 50 mM Tris buffer pH 7.5.

Cell layers were washed with wash buffer (10 mM Tris buffer (pH 7.5), 5mM MgCl, 150 mM NaCl), scraped in an homogenising buffer (10 mM Tris buffer (pH 7.5), 250 mM Sucrose, 1 mM EDTA, 5 mM MgCl, 150 mM NaCl, and 1x protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), passed through a 25 gauge needle, and homogenised on ice with 30-40 cycles in a Dounce homogeniser.

The homogenate was centrifuged for 10 min at 2500 x g at 4°C. The resulting supernatant was centrifuged for 90 min at 45000 x g at 4°C. The

membrane-enriched pellet was solubilised for 1 h in solubilising buffer (10 mM Tris buffer (pH 7.5), 5 mM MgCl, 150 mM NaCl, 1% Triton–X100, 1x protease inhibitor cocktail (Roche, see above)). Soluble membrane proteins were collected after further centrifugation for 1 hour at 45000 x g at 4°C.

Where rCTGF/V5 fusion protein was used, CTGF-cross linked proteins were either immunoprecipitated with rabbit anti CTGF antibody, or captured on a Pull-Down PolyHis column (Pierce Biotechnology, Tattenhall, Cheshire, UK).

Where rCTGF was used, CTGF-cross linked proteins were captured on a goat anti–CTGF-C terminal domain–Sepharose immunoaffinity column, using an IgG-Sepharose column as a control (FibroGen Inc.). After extensive washing of the columns with solubilising buffer, bound proteins were solubilised in reducing SDS-PAGE loading buffer, boiled for 5 min and resolved on 4-12% gradient gels by SDS-PAGE. Gels were either stained with Coomassie blue, or were used for Western blotting.

RNA extraction and RT-PCR analysis

Total RNA was extracted from 6 x 10^6 mesangial cells using the RNAzol B method (AMS Biotechnology (UK) Ltd., Oxfordshire, UK). Equal amounts of total RNA (2 µg) from each sample were reverse transcribed into cDNAs using SuperScript II RNase H+ reverse transcriptase (Gibco BRL, Paisley, Scotland, UK) and random primers.

Equal amounts (0.5 µl) of the reverse transcription reaction (20 µl) were subjected to PCR amplification in a 100 µl volume containing 10 µl of 10 x PCR buffer, 16 µl dNTPs (1.25 mM each), 2 mM MgCl2, 5 M betaine (Sigma), 0.5 µM of each specific primer and 1.25 U Amplitaq DNA

polymerase (Gibco BRL). Amplification was started with 5 min of denaturation at 94°C followed by 30 PCR cycles for all genes. Each cycle consisted of 60 s at 94°C, 60 s at 55°C and 60 s at 72°C. The final extension was for 10 min at 72°C. The sequences of primers to amplify TrkA, TrkB and TrkC $p75^{NTR}$, NGF, BDGF were as described by Anderson *et al.* (2002) *J. Clin. Endocrinol. Metab.* **87** pp. 890-897 and shown in Table 1 (adapted from Table 1 in Anderson et al. (2002)).

TABLE 1

Target	Primer	Sequence 5'-3'
TrkA	Forward	TCTTCACTGAGTTCCTGGAG
TrkA	Reverse	TTCTCCACCGGGTCTCCAGA
TrkB	Forward	AGTCCAGACACTCAGGATTTGTAC
TrkB	Reverse	CTCCGTGTGATTGGTAACATG
trTrkB	Forward	CATGTTACCAATCACACGGAGTA
trTrkB	Reverse	CCATCCAGTGGGATCTTATGAAA
TrkC	Forward	CATCCATGTGGAATACTACC
TrkC	Reverse	TGGGTCACAGTGATAGGAGG

Western blotting

Cells were lysed in reducing SDS-PAGE loading buffer and immediately scraped off the plate. Cell lysates were sonicated for 10 seconds to shear the DNA. Samples were then boiled for 5 minutes and resolved on 4-12% gradient gels by SDS-PAGE. Proteins were transferred onto a polyvinylidene difluoride membrane filter (Immobilin-P, Millipore, Bedford, UK) using a BioRad transfer apparatus. Blots were incubated in blocking buffer containing 1x TBS, 0.1% Tween-20 with 5% (w/v) non-fat dry milk, for 1 h.

Immunodetection was performed by incubating the blots in primary antibody at the appropriate dilution in antibody dilution buffer (1x TBS, 0.1% Tween-20 with 5% BSA), overnight at 4°C. Blots were then washed 3 times with washing buffer (1x TBS, 0.1% Tween-20) and incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies for 1 h at room temperature.

Bound antibodies were visualised using the enhanced chemi-luminescence reagent Luminol (Autogen Bioclear UK Ltd, Wiltshire, UK). Pre-stained molecular weight standards (Amersham International PLC, Amersham, UK) were used to monitor protein migration.

Immunofluorescence staining

Cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Coverslips were then incubated overnight at 4°C with serum (5% in PBS) from the same species as that in which the secondary antibody was raised. After the first incubation, the coverslips were incubated with primary antibodies (at optimum dilution in PBS containing 3% BSA) for 1 h at 37°C.

The coverslips were then washed and incubated in the dark for 1 h with fluorescein-conjugated secondary antibody (Sigma Aldrich, Dorset, UK). After staining, the coverslips were mounted on glass slides with anti-fade mounting media (Vector Labs, Peterborough, U.K.) and examined using a fluorescence microscope.

Results

CTGF activates several intracellular signalling pathways

Purified rCTGF-V5 fusion protein was used to identify the intracellular signal pathways which are activated in response to the growth factor in HMC. CTGF was found to rapidly trigger the activation of the classical MAPK (ERK1/2) and JNK pathways (Figures 1A and B) but not the p38 MAPK. Figure 1 shows the maximal activation of these kinases after 15 min of CTGF stimulation.

CTGF stimulation also led to the activation of Akt, also known as protein kinase B (PKB), at both the known phosphorylation sites; Thr-308 and Ser-473 (Figure 1C). The activation of Thr-308 appears to be rapid and sustained in comparison to the activation of ser- 473.

Activation of Thr-308 is influenced by a phosphoinositide-dependent kinase 1, or PDK1, whose activity is strictly dependent on 3-phosphorylated inositol lipids (Downward J (1998) Curr. Opin. Cell Biol. 10 pp/262-267). Phosphorylation of Ser-473 is conducted by integrin-linked kinase (ILK) (Attwell *et al.* (2000) *Oncogene* **19** pp. 3811-3815), and appears to be transient with a maximal level at 15 min, and return to a level close to the basal one within 30 min of CTGF exposure.

CTGF stimulation also led to the transient activation of Cam KII (Figure 1C). Other kinases which are activated in response to CTGF are PKC δ and PKC α (Figure 1D).

CTGF interaction with a receptor

Figure 1 demonstrates that CTGF provides a signal to downstream signalling proteins through a receptor that activates the above-mentioned kinases. These kinases are normally activated by a receptor tyrosine kinase (RTK).

The possibility of CTGF acting through a receptor tyrosine kinase (RTK) was tested by exposing HMC to CTGF for different periods of time. Cell lysates were prepared from the HMC cells and Western Blot analysis performed using an anti-phospho-tyrosine antibody.

The results showed that CTGF fusion protein (40 ng/ml) stimulated tyrosine phosphorylation within 10 min of at least two major proteins with apparent MW of about 75-80 and 140-150 kDa in HMC (Figure 2).

Another phosphotyrosine protein (MW 45 kDa) was detected in control cell lysates but was reduced in response to the CTGF treatment.

CTGF interacts with Human Mesangial Cell (HMC) surface proteins

The interaction of CTGF with HMC surface proteins was investigated by allowing CTGF to bind to the cell surface. A subsequent cross-linking procedure was performed and a membrane-enriched fraction isolated from the cells. After solubilisation this fraction was immunoprecipitated with a rabbit-anti-CTGF antibody. Covalently linked CTGF complexes were then analysed by PAGE and Western blotting with a chicken anti-CTGF antibody.

In Figure 3, CTGF appears to be cross-linked with membrane proteins to form complexes of apparent molecular weight 85 kDa, 180 kDa and >220 kDa, the latter being a large diffused band (lane 2). These complexes were not immunoprecipitated from the membrane-enriched fraction when the cross-linking step was eliminated (lane 1).

To ascertain whether CTGF activates an RTK a serum-starved HMC was incubated in both the presence and absence of CTGF for 15 min. The cells were lysed and phospho-tyrosine proteins immunoprecipitated. The immunoprecipitated proteins were analysed by Western blotting using antibodies against a plurality of known tyrosine kinase receptors.

Figure 4A shows the cross-reacted anti-TrkA antibody (a band of about 140 kDa). The intensity of this band was stronger when cells were incubated with CTGF (lane 2), indicating activation by CTGF.

The interaction of CTGF with the TrkA receptor was confirmed by different experiments in which either His-tagged CTGF/V5 fusion protein, or rCTGF expressed in the baculovirus system, was allowed to bind to the cell surface and then cross-linked to its ligand(s) using the reversible cross-linker DTSSP. The latter is then cleaved by reducing agents.

Subsequently, a membrane fraction was prepared and any cross-linked CTGF complexes were captured on affinity metal beads, or on anti-C-terminus CTGF antibody affinity beads. The captured complexes were subjected to SDS-PAGE under reducing conditions and analysed by Western blots.

Figures 4B, 4C, and 4D demonstrate that CTGF interacts with the TrkA receptor.

Trk receptors have previously been shown to interact with the pan neurotrophin receptor p75NTR. Therefore, blots were stripped and re-probed using an anti-p75NTR antibody. Figure 4E shows that the antibody cross-reacted with a protein of the correct molecular weight for P75NTR.

Thus the results demonstrate that CTGF interacts with the TrkA and p75NTR receptors.

HMC express Trk receptors

The expression of Trk receptors by HMC was investigated by extraction of total RNA from HMC for RT-PCR analysis to be performed on. Figure 5 shows that HMC express all three members of the Trk receptor family: TrkA, TrkB, and TrkC, as well as the pan receptor p75NTR.

CTGF activates TrkA in HMC

TrkA autophosphorylates several tyrosine residues on binding by its ligand, leading to the association and activation of multiple effector molecules. Phosphorylation at Tyr490 is required for Shc association and activation of the Ras-MAP kinase cascade. Phosphorylations at Tyr674/675 lie within the catalytic domain and reflect Trk kinase activity. Therefore we tested whether stimulating cells with CTGF leads to the phosphorylation of TrkA at these residues. The results in Figure 6 clearly indicate that CTGF induces the phosphorylation of the receptor at these residues.

Inhibition of CTGF-induced signalling by K252a

K252a is an alkaloid-like kinase inhibitor known to selectively inhibit tyrosine kinases. K252a blocked the protein phosphorylation of ERK1/2, JNK and ERK5 in HMC cells stimulated with CTGF. This indicted that the phosphorylation of these kinases is induced by the tyrosine kinase receptor, trkA, and that a tyrosine kinase inhibitor is capable of inhibiting CTGF mediated signalling.

CTGF induces expression of TIEG

Figure 7 shows that CTGF exposure causes a rapid increase in the expression level of TIEG.

The ability of TIEG to directly mediates the CTGF-dependent down-regulation of Smad 7 levels was investigated by treating cells with TIEG antisense and control oligonucleotides. Figure 8 shows that the constitutive levels of both TIEG and Smad 7 proteins in HMC are low (lane 1).

Incubating the cells with CTGF for 24 hours markedly increases the TIEG level whilst reducing Smad 7 to an almost undetectable level (lane 2). This effect is completely abolished in the presence of TIEG antisense oligonucleotide (lane 5), but not by the control oligonucleotide (lane 6).

Incubating the cells with TGF-b alone for the same period of time led to a moderate increase of both TIEG and Smad 7 (lane 3). However, incubating the cells with TGF-b in the presence of CTGF antisense oligonucleotide completely abolished the moderate induction of TIEG and led to the increased induction of Smad 7 (lane 7). This was not observed in the presence of the control antisense oligonucleotide (lane 8) and is consistent with TGF-b-induced CTGF being responsible for the observed moderate increase in TIEG expression level.

Similar results were also obtained by treating the cells with TIEG antisense and control antisense oligonucleotides (lanes 9 and 10). Incubating the cells with both TGF-b and CTGF (lane 4) markedly increases the expression level of TIEG whilst reducing the expression level of Smad 7. These results clearly show that TIEG mediates CTGF-dependent down-regulation of Smad 7 expression.

Example 2 – Screening method for identifying compounds inhibiting CTGF induced fibrosis

Screening for compounds possessing fibrosis inhibitory properties dependent on the CTGF-CTGF receptor interaction is conducted by testing the ability of each compound to block, for example, the induction of TIEG in HMC treated with CTGF.

The screening method is conducted using human mesangial cells (HMC) pre-incubated for 30 minutes with or without the potential inhibitor. These cells are then stimulated with CTGF-V5 fusion protein (40 ng/ml) in the presence or absence of the potential inhibitor for 2 hours. After washing the cell layer with cold PBS, the cells are lysed in RIPA buffer and the lysate assayed for TIEG by ELISA.

For the ELISA assay (Voller A *et al.*, (1976) in Manual of Clinical Immunology (Rose, N and Fishman H, eds.) pp 506-512, American Society of Microbiology, Washington, DC.), NUNC microtitre plates are coated overnight at 4°C with either lysate or with standard dilutions of r-TIEG to provide a standard curve.

After removing the coating solutions and washing the wells briefly with PBS, non-specific proteins are blocked by incubating the wells for 1 hour with1% (w/v) bovine serum albumin in PBS at 37°C. Wells are then incubated with anti-TIEG antibody at optimal dilution for 60 minutes, followed by peroxidase conjugated secondary antibody for 60 minutes at 37°C. After washing the wells three times with PBS, bound antibody is detected with the substrate 2,2'-azinobis-3-ethylbenzthazoline 6-sulphonic acid and absorbance read at 405 nm.

Recombinant TIEG protein is created from full length TIEG cDNA by cloning into the PcDNA 3.1/V5-His Topo vector (InVitrogen). This vector can be transfected into a mammalian cell line to express TIEG-fusion protein.

The TIEG fusion protein is purified from cell lysates using probond nickel-chelating resin. Anti-TIEG antibody is available from Dr. Steven Johnson (Mayo Foundation, Minnesota, USA) or can be raised in rabbits against the TIEG fusion protein using conventional methods.

Example 3 - Pharmaceutical formulations and administration.

The compounds of the invention will normally be administered orally or by any parenteral route, in the form of a pharmaceutical formulation comprising the active ingredient, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in a pharmaceutically acceptable dosage form. Depending upon the disorder and patient to be treated, as well as the route of administration, the compositions may be administered at varying doses.

In human therapy, the compounds of the invention can be administered alone but will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

For example, the compounds of the invention can be administered orally, buccally or sublingually in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed- or controlled-release applications. The compounds of invention may also be administered *via* intracavernosal injection.

Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxy-propylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

The compounds of the invention can also be administered parenterally, for example, intravenously, intra-arterially, intraperitoneally, intrathecally, intraventricularly, intrasternally, intracranially, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

For oral and parenteral administration to human patients, the daily dosage level of the compounds of the invention will usually be from 1mg/kg to 30 mg/kg. Thus, for example, the tablets or capsules of the compound of the invention may contain a dose of active compound for administration singly or two or more at a time, as appropriate. The physician in any event will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular

patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention.

The compounds of the invention can also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, *e.g.* dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A3 or 1;1,1,2,3,3,3-heptafluoropropane (HFA 227EA3), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or suspension of the active compound, *e.g.* using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, *e.g.* sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

Aerosol or dry powder formulations are preferably arranged so that each metered dose or "puff" delivers an appropriate dose of a compound of the invention for delivery to the patient. It will be appreciated that the overall daily dose with an aerosol will vary from patient to patient, and may be administered in a single dose or, more usually, in divided doses throughout the day.

Alternatively, the compounds of the invention can be administered in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. The compounds of the invention may also be transdermally administered, for example, by the use of a skin patch. They may also be administered by the ocular route, particularly for treating diseases of the eye.

For ophthalmic use, the compounds of the invention can be formulated as micronised suspensions in isotonic, pH adjusted, sterile saline, or, preferably, as solutions in isotonic, pH adjusted, sterile saline, optionally in combination with a preservative such as a benzylalkonium chloride. Alternatively, they may be formulated in an ointment such as petrolatum.

For application topically to the skin, the compounds of the invention can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, they can be formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Generally, in humans, oral or topical administration of the compounds of the invention is the preferred route, being the most convenient. In circumstances where the recipient suffers from a swallowing disorder or from impairment of drug absorption after oral administration, the drug may be administered parenterally, *e.g.* sublingually or buccally.

For veterinary use, a compound of the invention is administered as a suitably acceptable formulation in accordance with normal veterinary practice and the veterinary surgeon will determine the dosing regimen and route of administration which will be most appropriate for a particular animal.

CLAIMS

1. A method for identifying and/or making compounds for use in reducing and/or preventing fibrosis, comprising the steps:

(a) providing a CTGF receptor;

(b) providing a test sample;

(c) providing a CTGF receptor agonist;

(d) exposing the CTGF receptor to the test sample;

(e) subsequently or simultaneously exposing the CTGF receptor to the CTGF receptor agonist;

(f) detecting and/or measuring the amount of CTGF receptor activation;

(g) comparing the amount of CTGF receptor activation in the presence of a test sample with the amount of CTGF receptor activation detected and/or measured in the absence of a test sample; and

(h) determining if a compound reduces and/or prevents fibrosis on the basis that it causes no increase or a decrease in CTGF receptor activation.

2. The method of Claim 1 further comprising the step of:

(i) isolation of the compound capable of reducing and/or preventing fibrosis.

3. The method of Claim 2 further comprising the step of

(j) formulating the isolated compound into a composition further comprising a pharmaceutically acceptable carrier, excipient and/or diluent.

4. The method of any previous claim wherein CTGF receptor activation is measured and/or detected by detecting and/or measuring at least one of the following activities: CTGF receptor autophosphorylation; receptor-induced protein phosphorylation; and/or CTGF receptor induced TIEG expression.

5. The method of any previous claim wherein the CTGF receptor agonist is CTGF.

6. The method of any previous claim wherein the CTGF receptor is the TrkA receptor.

7. The method of any previous claim wherein the compound affects directly with the interaction between the CTGF receptor and an agonist thereof.

8. The method of any of claims 1 to 6 wherein the compound affects indirectly with the interaction between the CTGF receptor and an agonist thereof.

9. The method of any previous claim wherein the compound is a CTGF receptor antagonist.

10. A compound for use in the reduction and/or prevention and/or diagnosis of fibrosis characterised in that it inhibits and/or prevents CTGF receptor activation.

11. A compound identified and/or made by the method of any one of Claims 1 to 9 for use in the reduction and/or prevention and/or diagnosis of fibrosis.

12. A compound as claimed in either of Claims 10 and 11 which is at least one selected from polypeptides, antibody molecules, antisense nucleotides.

13. A compound as claimed in Claim 12 wherein the compound is an antibody molecule.

14. A compound as claimed in Claim 12 wherein the compound is a CTGF receptor antagonist.

15. Use of a compound identified and/or made by any of Claims 1 to 9 in the treatment and/or prevention and/or diagnosis of a fibrotic disease.

16. Use of a compound identified and/or made by any of Claims 1 to 9 in the manufacture of a medicament for the treatment and/or prevention and/or diagnosis of a fibrotic disease.

17. Use of a compound as claimed in either Claim 10 or 11 in the treatment and/or prevention and/or diagnosis of a fibrotic disease.

18. Use of a compound as claimed in either Claim 10 or 11 in the manufacture of a medicament for the treatment and/or prevention and/or diagnosis of a fibrotic disease.

19. A use as claimed in any one of Claims 15 to 18 wherein the fibrotic disease is selected from one or more diabetic nephropathy, no-diabetic kidney fibrosis, lung fibrosis, liver fibrosis (cirrhosis), skeletal muscle fibrosis, cardiac muscle fibrosis, atherosclerosis, systemic sclerosis, scleroderma, retinal fibrosis, radiation induced fibrosis keloid scar formation and cancer-associated fibrosis.

20. A use as claimed in Claim 19 wherein the disease is diabetic nephropathy.

21. A method of treating and/or preventing a fibrotic disease comprising administering a therapeutically or prophylactically effective dose, or plurality of doses, of a compound identified and/or made by the method of any of Claims 1 to 9.

22. A method of treating and/or preventing a fibrotic disease comprising administering a therapeutically or prophylactically effective dose, or plurality of doses, of a compound as claimed in any of Claims 10 to 14.

23. A method as claimed in either Claim 21 or 22 wherein the fibrotic disease is selected from one or more diabetic nephropathy, no-diabetic kidney fibrosis, lung fibrosis, liver fibrosis (cirrhosis),

skeletal muscle fibrosis, cardiac muscle fibrosis, atherosclerosis, systemic sclerosis, scleroderma, retinal fibrosis, radiation induced fibrosis keloid scar formation and cancer-associated fibrosis.

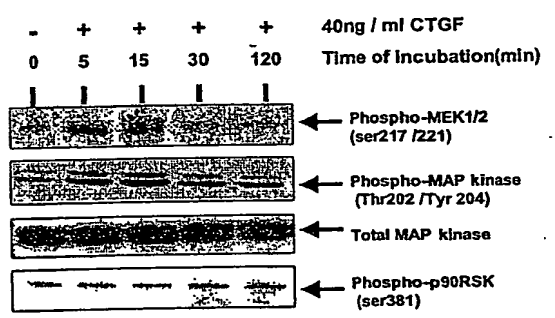
24. A method as claimed in Claim 23 wherein the fibrotic disease is diabetic nephropathy.

ABSTRACT

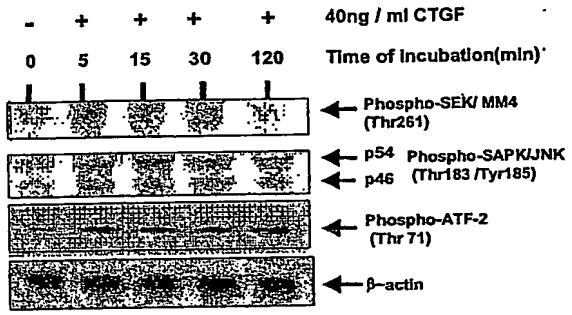
BIOLOGICAL MATERIALS AND USES THEREOF

The invention provides a method for identifying and/or making compounds for use in reducing and/or preventing fibrosis, comprising the steps: providing a CTGF receptor; providing a test sample; providing a CTGF receptor agonist; exposing the CTGF receptor to the test sample; subsequently or simultaneously exposing the CTGF receptor to the CTGF receptor agonist; detecting and/or measuring the amount of CTGF receptor activation; comparing the amount of CTGF receptor activation detected and/or measured in the presence of the test sample with the amount of CTGF receptor activation detected and/or measured in the absence of a test sample; determining if a compound reduces and/or prevents fibrosis on the basis that it causes no increase or a decrease in CTGF receptor activation. There is also provided compounds for reducing and/or preventing fibrosis and uses of such compounds.

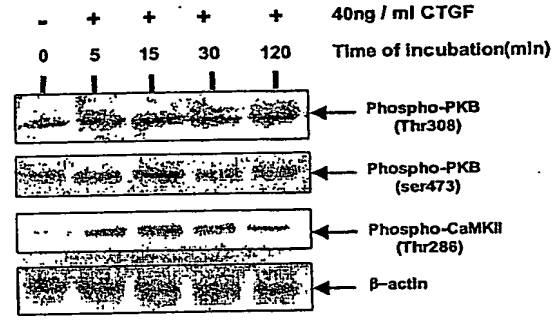
Figure. 1


- + + + + 40ng / ml CTGF
0 5 15 30 120 Time of Incubation(min)
Phospho-MEK1/2 (ser217 /221)
Phospho-MAP kinase (Thr202 /Tyr 204)
Total MAP kinase
Phospho-p90RSK (ser381)

A)


- + + + + 40ng / ml CTGF
0 5 15 30 120 Time of Incubation(min)
Phospho-SEK/ MM4 (Thr261)
p54 Phospho-SAPK/JNK
p46 (Thr183 /Tyr185)
Phospho-ATF-2 (Thr 71)
β-actin

B)


- + + + + 40ng / ml CTGF
0 5 15 30 120 Time of Incubation(min)
Phospho-PKB (Thr308)
Phospho-PKB (ser473)
Phospho-CaMKII (Thr286)
β-actin

C)

Figure 1

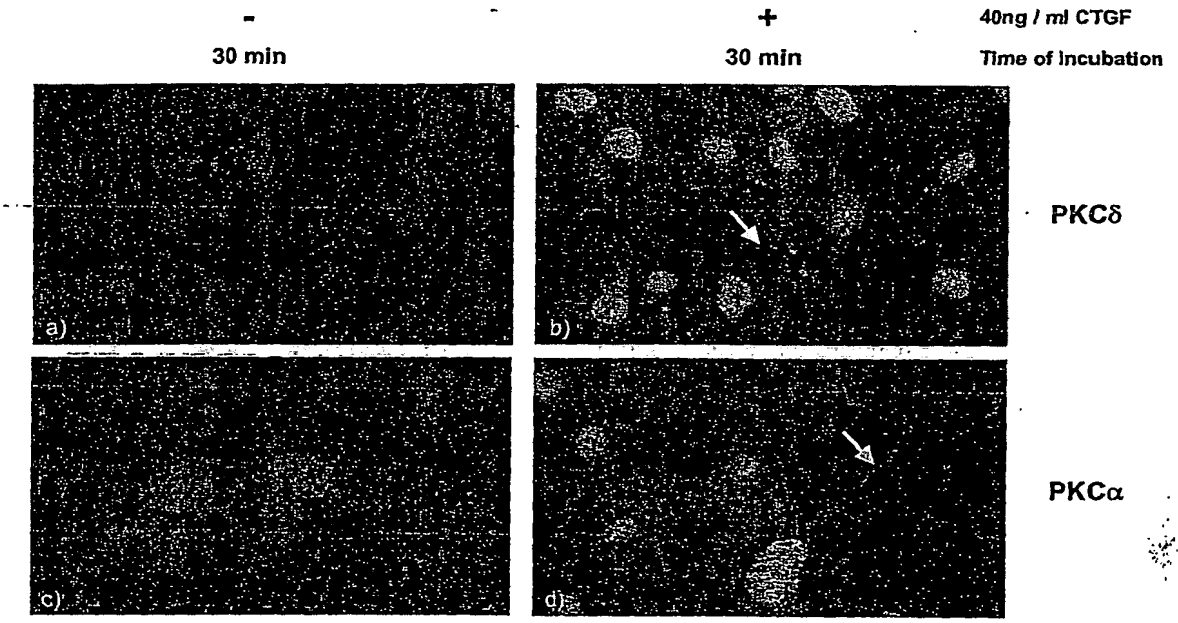


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Figure 1 (con't)

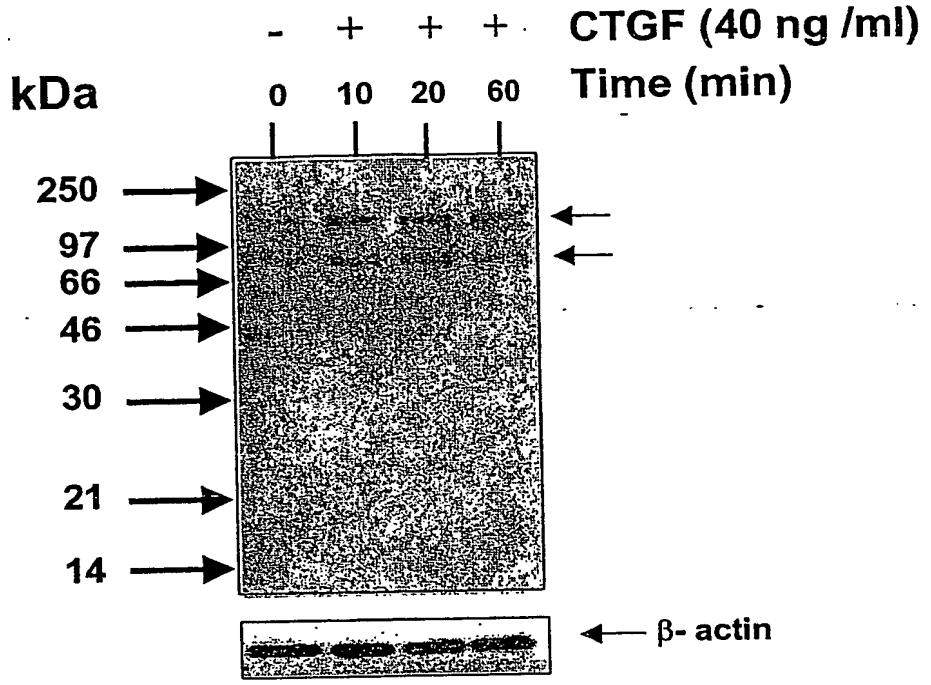


Figure 2

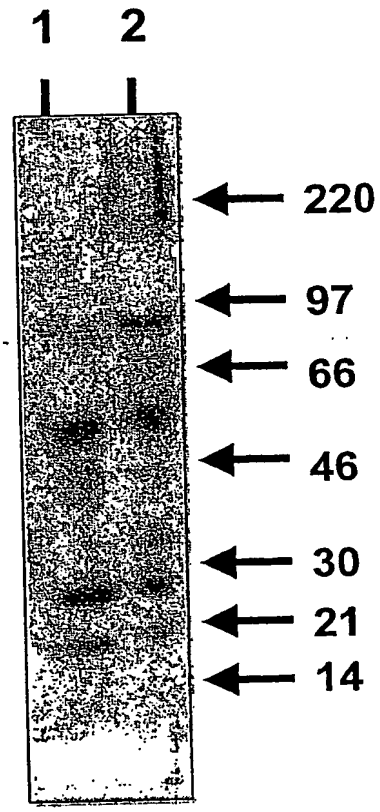
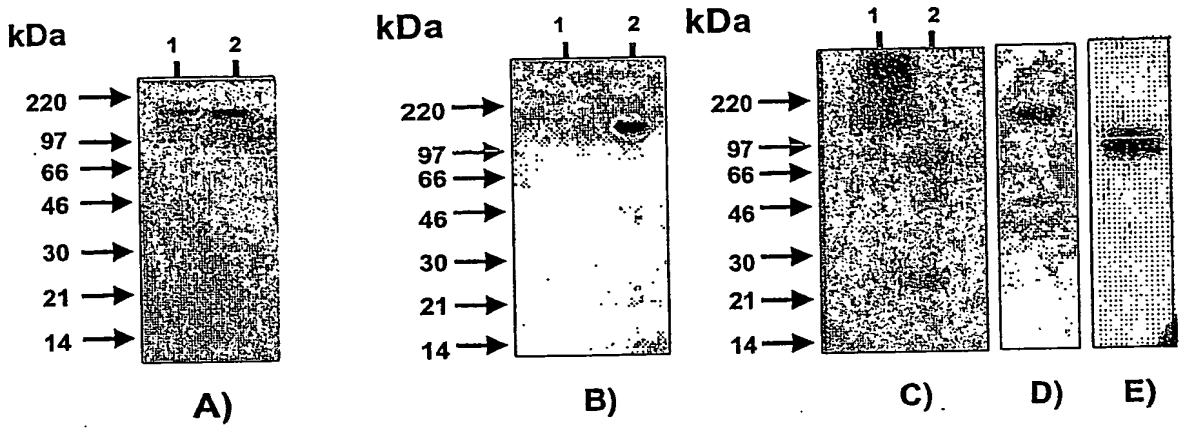


Figure 3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E)

Figure 4

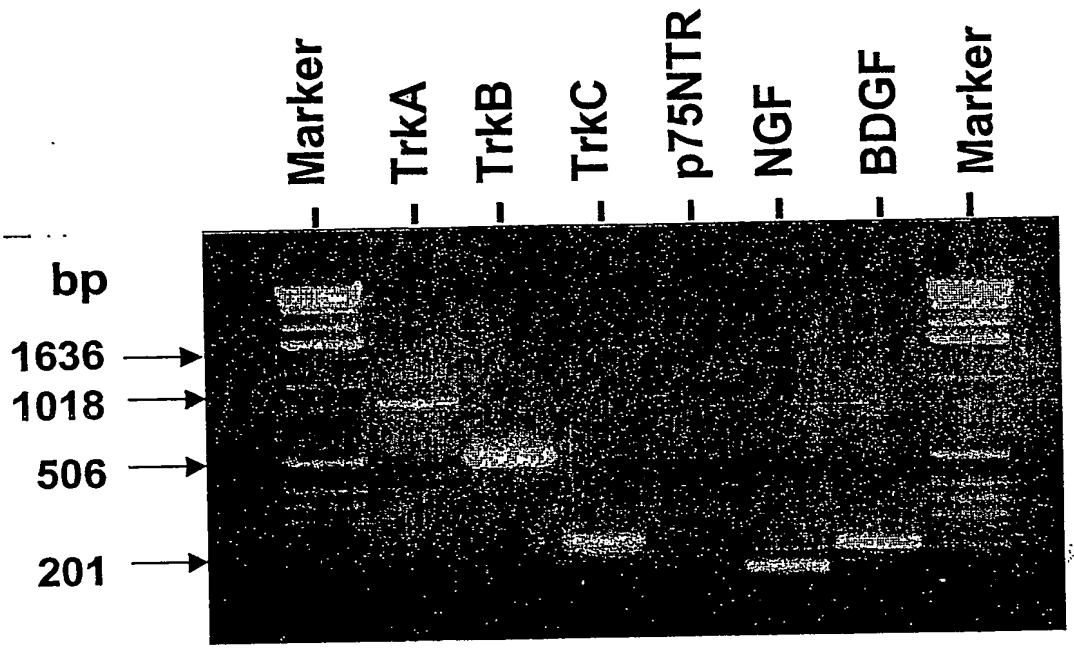
Marker
TrkA
TrkB
TrkC
p75NTR
NGF
BDGF
Marker
bp
1636
1018
506
201

Figure 5

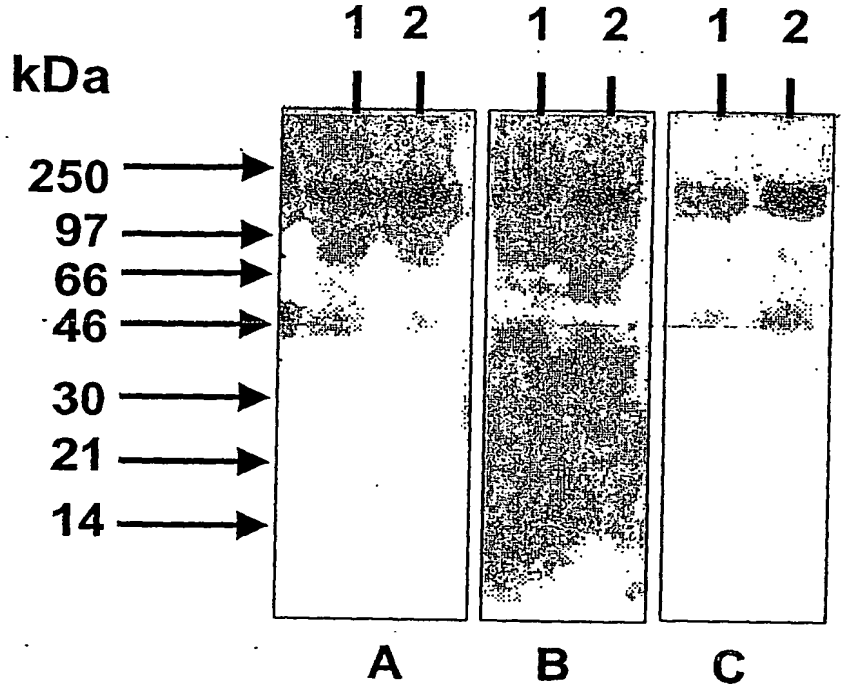

Figure 6

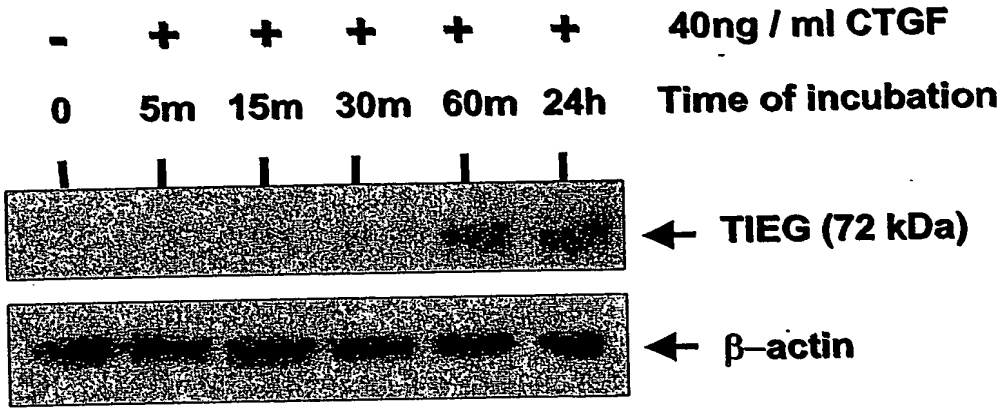
- + + + + +
40ng / ml CTGF
0 5m 15m 30m 60m 24h
Time of incubation
TIEG (72 kDa)
β–actin

Figure 7

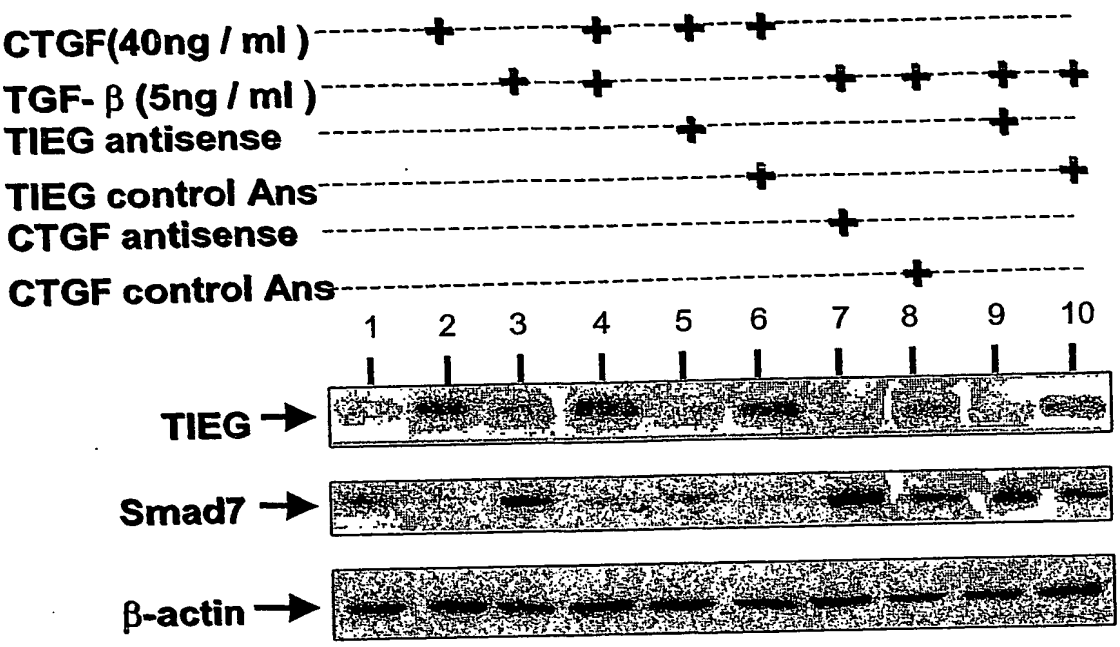
CTGF(40ng / ml)
TGF- β (5ng / ml)
TIEG antisense
TIEG control Ans
CTGF antisense
CTGF control Ans
1
2
3
4
5
6
7
8
9
10
TIEG
Smad7
β-actin

Figure 8